

## Molybdoenzyme Biosynthesis in *Escherichia coli*: In Vitro Activation of Purified Nitrate Reductase from a *chlB* Mutant

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**All molybdoenzyme activities are absent in *chlB* mutants because of their inability to synthesize molybdopterin guanine dinucleotide, which together with molybdate constitutes the molybdenum cofactor in *Escherichia coli*. The *chlB* mutants are able to synthesize molybdopterin. We have previously shown that the inactive nitrate reductase present in a *chlB* mutant can be activated in a process requiring protein FA and a heat-stable low-molecular-weight substance. We show here that purified nitrate reductase from the soluble fraction of a *chlB* mutant can be partially activated in a process that requires protein FA, GTP, and an additional protein termed factor X. It appears that the molybdopterin present in the nitrate reductase of a *chlB* mutant is converted to molybdopterin guanine dinucleotide during activation. The activation is absolutely dependent upon both protein FA and factor X. Factor X activity is present in *chlA*, *chlB*, *chlE*, and *chlG* mutants.**

All known molybdoenzymes other than the dinitrogenases contain a molybdenum cofactor which consists of a pterin derivative, molybdopterin, complexed with molybdate (11, 16, 17). In certain microorganisms, including *Escherichia coli*, the molybdenum cofactor also contains a GMP moiety attached to molybdopterin to form molybdopterin guanine dinucleotide (14, 15, 18).

In *E. coli*, mutations in the five chlorate resistance loci (*chlA*, *chlB*, *chlD*, *chlE*, and *chlG*) lead to the pleiotropic loss of all molybdoenzyme activities in the bacterium (4, 29). The *chlA* and *chlE* loci encode functions required for molybdopterin biosynthesis (19, 24), and *chlB* mutants lack molybdopterin guanine dinucleotide but possess molybdopterin, suggesting that the *chlB* locus is required for molybdopterin guanine dinucleotide biosynthesis (18). The *chlD* locus appears to be responsible for molybdate uptake (8, 13). The function of the *chlG* locus is unclear (29). All of the *chl* loci have been cloned (25), and most of these loci have been sequenced (13, 23). In the biosynthesis of molybdoenzymes, the sequence of action of these gene products in the post-translational events of cofactor acquisition remains to be established.

Incubating crude soluble extracts of *chlA* and *chlB* mutants together leads to biochemical complementation and the formation of molybdoenzyme, notably nitrate reductase, activities (1). Fractionation of the soluble fraction of a *chlA* mutant resulted in a protein preparation containing protein FA, which when added to the soluble fraction brought about molybdoenzyme activation (6, 26). Since protein FA is actively expressed in all classes of *chl* mutants except *chlB* mutants, it is probably the product of the *chlB* gene (21). It is constitutively expressed in *E. coli*. Protein FA has been purified from the soluble fraction of a *chlA* mutant and its apparent relative molecular mass was about 18,000 (26, 27). Further characterization of the protein FA-dependent activation of the inactive nitrate reductase present in the soluble fraction of a *chlB* mutant showed that a heat-stable low-

molecular-weight substance distinct from molybdenum cofactor was required (5).

An important goal in this work is to achieve the in vitro activation of nitrate reductase from *chlB* mutants with defined purified components. In this study, we have further characterized the protein FA-dependent activation of nitrate reductase from a *chlB* mutant and have demonstrated activation of the nitrate reductase after its purification to apparent homogeneity. We show that GTP can replace the requirement for the heat-stable low-molecular-weight material. An additional protein, factor X, in addition to protein FA, is required for activation.

### MATERIALS AND METHODS

**Strains.** The strains used in this work are listed in Table 1.

**Construction of the double-mutant strains.** The *chl::Mucts nar::Mucts* double mutants used in this work were constructed as follows. A transposon cotransducible with the *chl* gene of interest was transduced in the corresponding *chl::Mucts* strain (29). A P1 lysate grown on the resulting strain was used to cotransduce the *chl* allele with the transposon into the LCB 162 strain (*nar::Mucts*). The expected double mutants, phenotypically deficient in molybdenum cofactor biosynthesis, were those of the selected transductants which were unable to produce gas via the formate-hydrogenlyase system. The following insertions were used: *metE163::Tn10*, cotransducible with *chlB*; *zbi::Tn10*, cotransducible at frequencies of 95% with *chlE*, 13% with *chlA*, and 1% with *chlD*; and *leu::Tn9*, cotransducible with *chlD*.

**Growth of bacteria and preparation of subcellular fractions.** The bacteria were grown in basal medium (1) with glucose (2 g/liter), yeast extract (Difco) (2 g/liter), Bacto Peptone (Difco) (2 g/liter), and 1  $\mu$ M Na molybdate; KNO<sub>3</sub> (1 g/liter) was added for expression of nitrate reductase. Anaerobic growth was accomplished in stoppered bottles filled almost to the top. Cultures were incubated at 37°C, unless the strain was a bacteriophage Mu (*Mucts*) lysogen and then the temperature was kept at 30°C. Cells were harvested at late exponential

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype	Reference
AP 601	F <sup>-</sup> <i>thr leu his pro arg thi ade gal lacY malE xyl ara mtl str</i> T1 <sup>r</sup> λ <sup>r</sup>	Boxer et al. (5)
AP 24	Same as AP 601, but <i>chlB</i>	Boxer et al. (5)
LCB 162	F <sup>-</sup> <i>thr leu lacY tonA thi str narG162::Mucts62</i>	Bonnefoy-Orth et al. (3)
LCB 162 A	Same as LCB 162, but <i>chlA203::Mucts</i>	This study
LCB 162 B	Same as LCB 162, but <i>chlB207::Mucts</i>	This study
LCB 162 E	Same as LCB 162, but <i>chlE201::Mucts</i>	This study
LCB 162 G	Same as LCB 162, but <i>chlG206::Mucts</i>	This study

phase and suspended after washes in a solution consisting of 50 mM Tris-HCl (pH 7.6) and 1 mM benzamidine-HCl. Bacteria were broken at 69 MPa by passage through a French press, and cellular debris was removed by centrifugation (18,000 × *g* for 15 min). The soluble fractions were obtained after further centrifugation (120,000 × *g* for 90 min). All operations were performed at 4°C.

**Purification of inactive nitrate reductase from a *chlB* mutant.** Detection and quantification of inactive nitrate reductase from strain AP 24, a *chlB* mutant, were performed by rocket immunoelectrophoretic analysis (9). Antiserum was raised in rabbits immunized with nitrate reductase A purified from a wild-type strain.

Cells (100 g [wet weight]) were suspended in 300 ml of 40 mM Tris-HCl (pH 7.6) containing 1 mM benzamidine-HCl. The crude extract (4,200 mg of protein) obtained after breakage of cells was centrifuged at 120,000 × *g* for 90 min. The soluble fraction (3,200 mg of protein), which contains nitrate reductase, was precipitated with ammonium sulfate (35% saturation), stirred at 4°C in a nitrogen atmosphere, and then centrifuged for 20 min at 18,000 × *g*. The precipitate (550 mg of protein) obtained was suspended in 12 ml of 40 mM Tris-HCl (pH 7.6) containing 1 mM benzamidine-HCl, desalted on a Sephadex G25 column (20 by 2.6 cm), and eluted in the same buffer. The eluate was applied to a DEAE Sepharose CL-6B column (80 by 2.6 cm) equilibrated with a solution containing 100 mM Tris-HCl (pH 7.6), 1 mM benzamidine, and 100 mM NaCl. One thousand milliliters of a 100 to 450 mM NaCl gradient in the same buffer was applied to the column. The fractions containing inactive nitrate reductase (eluted in the range of 0.26 to 0.28 M NaCl), were pooled and concentrated to 5 ml by membrane ultrafiltration with a Diaflo PM30 membrane. The sample (80 mg of protein) was applied to a Sephadex G<sub>150</sub> column (82 by 2.6 cm) that had been equilibrated with 40 mM Tris-HCl (pH 7.6) containing 1 mM benzamidine-HCl. The fractions containing nitrate reductase (27 mg of protein) were pooled, and purified by fast protein liquid chromatography on a Mono Q HR10/10 ion-exchange column equilibrated with the same buffer. Elution of sample was effected by applying a 0 to 400 mM NaCl gradient to the column.

Fractions containing purified nitrate reductase (15 mg) were pooled, dialyzed for 3 h against 40 mM Tris-HCl (pH 7.6)–1 mM benzamidine, freeze-dried, and kept at –80°C. At the end of the process, the enzyme had been purified 26-fold, with a yield of 11%.

Purified enzyme was subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide) and the protein was stained with Coomassie blue and possessed an *R<sub>f</sub>* of 0.23. Subunit composition of the purified enzyme was performed by Western blotting (immunoblotting), as described by Blasco et al. (2).

**Activation of purified nitrate reductase from a *chlB* mutant.**

The incubation mixtures routinely contained purified nitrate reductase (10 μg of protein), 30 μg of purified protein FA, 1 mM GTP, and 1 μM MgCl<sub>2</sub> in a minimum volume of 40 mM Tris-HCl (pH 7.6) to which factor X (0 to 200 μg of protein) had been added. The mixtures were incubated for up to 2 h at 32°C in a nitrogen atmosphere generally as originally described by Azoulay et al. (1). The mixtures were kept on ice until the start of activation, which was initiated by the addition of either factor X or protein FA. The total volume of the mixture was kept constant (500 μl) by adjustment of the buffer addition. Protein FA was prepared as described previously (26).

**Preparation of heated extracts.** Heated extracts from either the crude extract of a *chlB* mutant or a preparation of factor X were performed as described previously (28).

**Trypsin treatment of factor X.** A sample of partially purified factor X (80 μg) was incubated with 1 μg of trypsin (bovine pancreas type III [Sigma]) at 37°C for 2 h at pH 7.6. Trypsin was inactivated by the addition of 2 μg of soybean trypsin inhibitor (type II-S [Sigma]). To this mixture, purified nitrate reductase, protein FA, and GTP were added as described above. Controls were done by incubating trypsin with its inhibitor prior to factor X addition.

**Fluorescence analysis.** Purified nitrate reductase (1 mg) or partially purified factor X (10 mg) in 1 ml of deaerated 40 mM Tris-HCl (pH 7.6) was heated for 5 min at 100°C in a nitrogen atmosphere. The tubes were centrifuged at 18,000 × *g* for 15 min. Fluorescence analysis of the supernatants was performed by using a Kontron SFM25 spectrofluorimeter, as described by Giordano et al. (6).

**Enzyme assays and analytical procedures.** Nitrate reductase activities were measured spectrophotometrically (20) by the oxidation of reduced benzyl viologen, which leads to the reduction of nitrate to nitrite. One unit of nitrate reductase activity is the amount catalyzing the production of 1 μmol of nitrite min<sup>-1</sup>. Protein concentrations were estimated by the technique of Lowry et al. (22).

## RESULTS

**Purification and properties of inactive nitrate reductase from a *chlB* mutant.** The inactive nitrate reductase present in the soluble fraction of a *chlB* mutant was purified as described in Materials and Methods. Reasonable yields of the nitrate reductase derivative were attained, and in the better preparations, a 26-fold purification was achieved. SDS-PAGE analysis revealed two stained protein bands which were indistinguishable from those corresponding to the α- and β-subunits of the active enzyme (12) and which were recognized by nitrate reductase antibodies after Western immunoblotting (data not shown). As we showed previously for the wild-type nitrate reductase (6), the presence of pterin-like material in the enzyme was established by fluo-

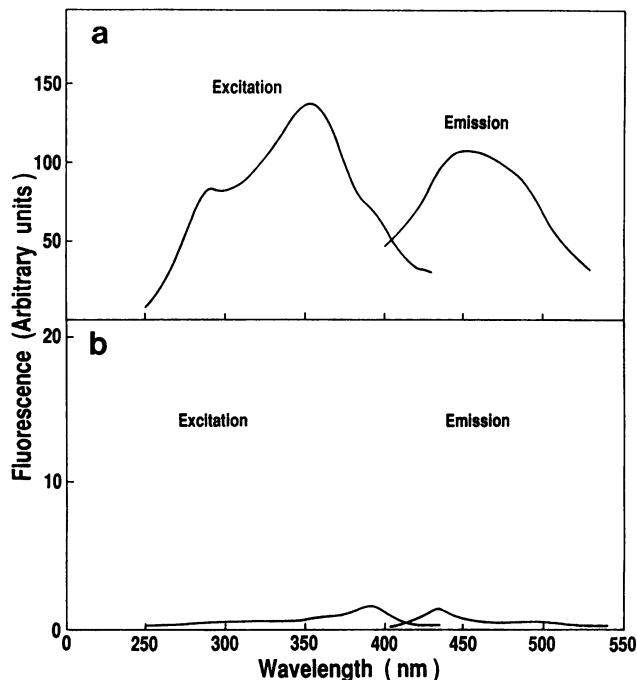


FIG. 1. Fluorescence analysis of nitrate reductase from a *chlB* mutant. (a) Purified nitrate reductase (1 mg of protein) was heated as described in Materials and Methods, adjusted to 0.1 M NaOH, deaerated, and subjected to fluorimetric analysis. For the excitation spectrum, fluorescence was measured at 455 nm. For the emission spectrum, irradiation was at 360 nm. (b) The preparation of factor X (10 mg of protein) was treated and analyzed exactly as described for panel a.

rescence analysis (Fig. 1). The presence of molybdopterin, but not molybdopterin guanine dinucleotide, in a *chlB* strain has recently been reported (18). Our finding is consistent with the presence of molybdopterin in *chlB* strains but also indicates that pterin-like material is incorporated into the inactive nitrate reductase in a *chlB* mutant.

**Purified nitrate reductase from a *chlB* mutant cannot be activated with protein FA and low-molecular-weight material.** We have previously shown that the activation of nitrate reductase in a *chlB* mutant requires protein FA and heat-stable low-molecular-weight material (5). The source of nitrate reductase in our earlier experiments was the soluble fraction from a *chlB* mutant after passage through a Sephadex G25 desalting column. If the activation process simply involved the direct action of protein FA and the low-molecular-weight material on the nitrate reductase from a *chlB* mutant, then it should be possible to substitute purified nitrate reductase from a *chlB* mutant for the desalted soluble fraction from a *chlB* mutant in the activation mixture. In many experiments of this kind, we were unable to achieve any activation of the purified nitrate reductase from a *chlB* mutant (Table 2).

This result strongly suggests that an additional component present in the desalted soluble fraction from a *chlB* mutant is required for the activation of nitrate reductase. Consistent with this view, activation of purified nitrate reductase from a *chlB* mutant could be achieved if a little of the soluble fraction of a *chlB narG* mutant was introduced into the activation mixture along with protein FA and the deproteinized heated extract from a *chlB* mutant (Table 2). The

TABLE 2. Activation of purified nitrate reductase from a *chlB* mutant

Addition <sup>a</sup>	Activity <sup>b</sup>
1. None .....	≤1
2. Heated soluble fraction from a <i>chlB</i> mutant.....	≤1
3. Soluble fraction of strain LCB 162 B ( <i>chlB narG</i> ).....	19
4. Same as 3, but with heated soluble fraction from a <i>chlB</i> mutant .....	102
5. Same as 3, but with 1 mM GTP .....	73
6. Same as 5, but with 1 μM MgCl <sub>2</sub> .....	89
7. Same as 5, but with 10 μM MgCl <sub>2</sub> .....	87

<sup>a</sup> The additions indicated were added to the standard activation mixture containing 10 μg of purified nitrate reductase from a *chlB* mutant and 30 μg of protein FA. When present, the amounts used were as follows: 300 μl of heated soluble fraction from a *chlB* mutant and 50 μl of soluble fraction of strain LCB 162 B (*chlB narG*).

<sup>b</sup> Nitrate reductase activity expressed as nanomoles of nitrate reduced per min per incubation mixture.

soluble fraction from a *chlB narG* mutant lacks both activatable nitrate reductase (*narG*) and protein FA activity (*chlB*) but clearly contains material required for the activation of nitrate reductase from a *chlB* mutant. This material must have been removed during the purification of nitrate reductase from a *chlB* mutant. Furthermore, this experiment showed that nitrate reductase from a *chlB* mutant can be isolated in a state capable of being activated.

Experiments have been performed with equivalent amounts of nitrate reductase derived from the unfractionated soluble fraction of a *chlB* mutant and nitrate reductase isolated from a *chlB* mutant in the activation mixtures, with the amount of nitrate reductase present from each source being monitored by rocket immunoelectrophoresis (9). From such experiments, we estimate that the nitrate reductase isolated from a *chlB* mutant can be activated to about 30% the level that can be achieved using the unfractionated soluble fraction from a *chlB* mutant (data not shown). This result suggests that after its isolation nitrate reductase from a *chlB* mutant has reduced ability to be activated.

**GTP greatly stimulates the activation of nitrate reductase from a *chlB* mutant.** The recent demonstration that *chlB* mutants lack molybdopterin guanine dinucleotide but possess molybdopterin suggests that the *chlB* locus encodes a function required for GMP attachment to molybdopterin. We therefore examined the effect of adding GTP to the activation mixture. We replaced the deproteinized heated extract with 1 mM GTP in the activation mixture for purified nitrate reductase, and activation was observed (Table 2).

The level of activation obtained with 1 mM GTP was over 70% that found when the heated extract from a *chlB* mutant was used. This result is consistent with the activation of nitrate reductase from a *chlB* mutant involving molybdopterin guanine dinucleotide formation and suggests that GTP may be the source of the GMP moiety. The active component in the heated soluble fraction from a *chlB* mutant is most probably GTP. Adding 1 μM MgCl<sub>2</sub> enhanced the activation observed with GTP (Table 2). The involvement of Mg<sup>2+</sup> in the activation process has been shown earlier (5).

**Identification of an additional component required for protein FA-dependent activation of nitrate reductase from a *chlB* mutant.** The experiments described above indicate that the soluble fraction of the *chlB narG* mutant contains component(s) required for nitrate reductase activation other than protein FA and GTP. In order to explore the nature of the additional component(s) required for nitrate reductase acti-

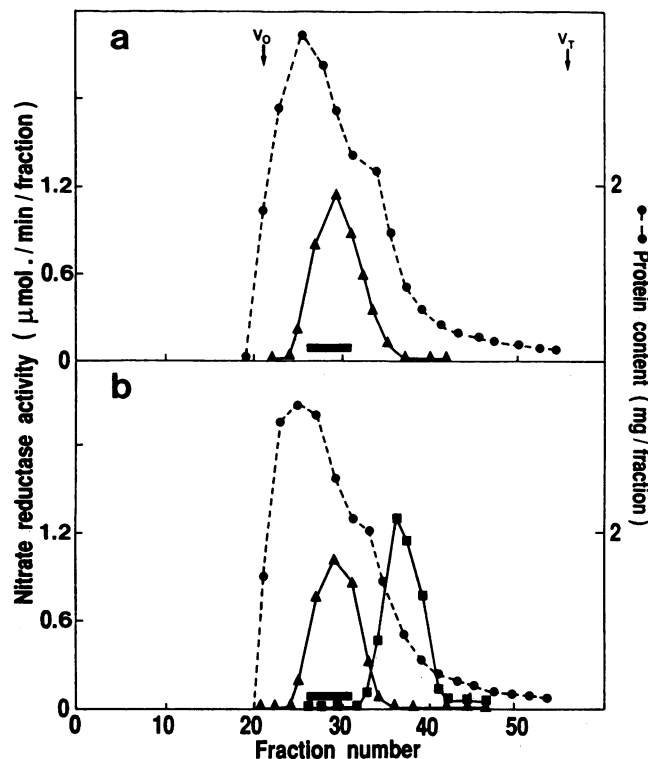


FIG. 2. Identification of a component required for the activation of nitrate reductase from a *chlB* mutant in addition to protein FA. The soluble fraction (50 mg of protein) from either strain LCB 162 B (*chlB narG*) (a) or strain LCB 162 A (*chlA narG*) (b) was prepared and applied to a Sephadex G75 column (1.6 by 60 cm) equilibrated and eluted with a solution containing 20 mM Tris-HCl (pH 7.6), 1 mM benzamidine, and 200 mM NaCl. (a) Fractions containing factor X activity ( $\blacktriangle$ ) were detected by their ability to promote the activation of purified nitrate reductase from a *chlB* mutant. Aliquots of the fractions (150  $\mu$ l) were incubated for 30 min with purified nitrate reductase (10  $\mu$ g), protein FA (30  $\mu$ g),  $MgCl_2$  (1  $\mu$ M), and GTP (1 mM) as described in Materials and Methods. The fractions containing factor X activity were combined as indicated, by the bar in the figure.  $V_0$  and  $V_T$  indicate the excluded and total column volumes, respectively. (b) Fractions containing factor X activity ( $\blacktriangle$ ) were assayed as described for panel a. Protein FA activity ( $\blacksquare$ ) was assayed by incubation of aliquots (200  $\mu$ l) with nitrate reductase from a *chlB* mutant (15  $\mu$ g), factor X (160  $\mu$ g) obtained from an experiment such as that described for panel a,  $MgCl_2$  (1  $\mu$ M), and GTP (1 mM).

vation, we subjected the soluble fraction from a *chlB narG* mutant to Sephadex G75 gel filtration and monitored the fractions for their ability to promote the activation of purified nitrate reductase from a *chlB* mutant in the presence of protein FA and GTP (Fig. 2a). A clear peak of activity was identified. When either GTP or protein FA was omitted from the assay mixture, no activity was found in the column fractions. The active material eluted from the column in a position distinct from both protein FA (Fig. 2b) and nitrate reductase, corresponding to a relative molecular mass in the 35,000 to 60,000 range. The active fractions were combined as indicated and used without further purification in the experiments described here. The component in the preparation was termed factor X.

**Characterization of factor X activity.** The progress of the activation of purified nitrate reductase from a *chlB* mutant

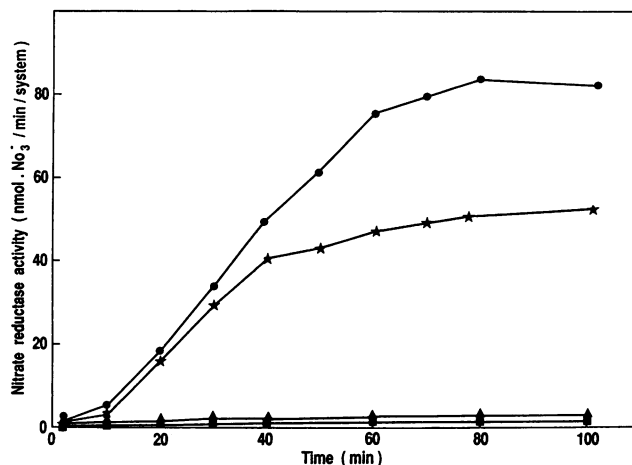


FIG. 3. Time course of activation of nitrate reductase from a *chlB* mutant in the presence of factor X. The standard activation mixture contained, in a final volume of 500  $\mu$ l, nitrate reductase from a *chlB* mutant (10  $\mu$ g), protein FA (30  $\mu$ g), factor X (80  $\mu$ g),  $MgCl_2$  (1  $\mu$ M), and GTP (1 mM). The mixture was incubated and subsequently assayed for nitrate reductase activity ( $\bullet$ ), as described in Materials and Methods. Nitrate reductase activity was also determined when less nitrate reductase (5  $\mu$ g) was used ( $\ast$ ), when factor X was heated at 100°C for 5 min ( $\blacktriangle$ ), and when factor X was exposed to trypsin, as described in Materials and Methods. Activities are expressed in nanomoles of nitrate reduced per min per reaction mixture.

brought about by factor X, protein FA, and GTP is shown in Fig. 3. Under the conditions used, the activity reaches a limiting level after approximately 60 min. The amount of nitrate reductase present, rather than any of the other participants in activation, limits the extent of the activation, since when less nitrate reductase from a *chlB* mutant was used, activation was proportionally reduced. After reactivation, the maximum specific activity observed for purified nitrate reductase from a *chlB* mutant (10.2  $\mu$ mol of nitrate reduced per min per mg of nitrate reductase) is 15% that for native nitrate reductase. This result, in accordance with the results above, suggest that *chlB* nitrate reductase is less activatable after purification.

The proteinaceous character of factor X was confirmed by the complete loss of its activity after exposure either to 100°C for 5 min or to the proteinase trypsin (Fig. 3). These findings indicate that factor X is unlikely to function in the activation by simply supplying a heat-stable compound which is bound to protein.

Molybdopterin or related derivatives could not be detected in factor X preparations after denaturation and fluorimetric analysis (Fig. 1).

The activation of nitrate reductase from a *chlB* mutant is absolutely dependent upon the combined presence of factor X, protein FA, and GTP (Fig. 4). The data in Table 3 show that 1 mM GTP with 1  $\mu$ M  $MgCl_2$  brings about the same level of activation as that found when the heated soluble fraction from a *chlB* mutant is used in the activation. High levels of GTP (5 mM) were found to inhibit activation. GDP was almost as effective as GTP in the system, but GMP produced only 22% activation. Of the other nucleotides tested, only ATP gave rise to a low level of activation. The likely presence in factor X preparations of some nucleotide transferase or guanylate kinase activities could explain some of these findings.

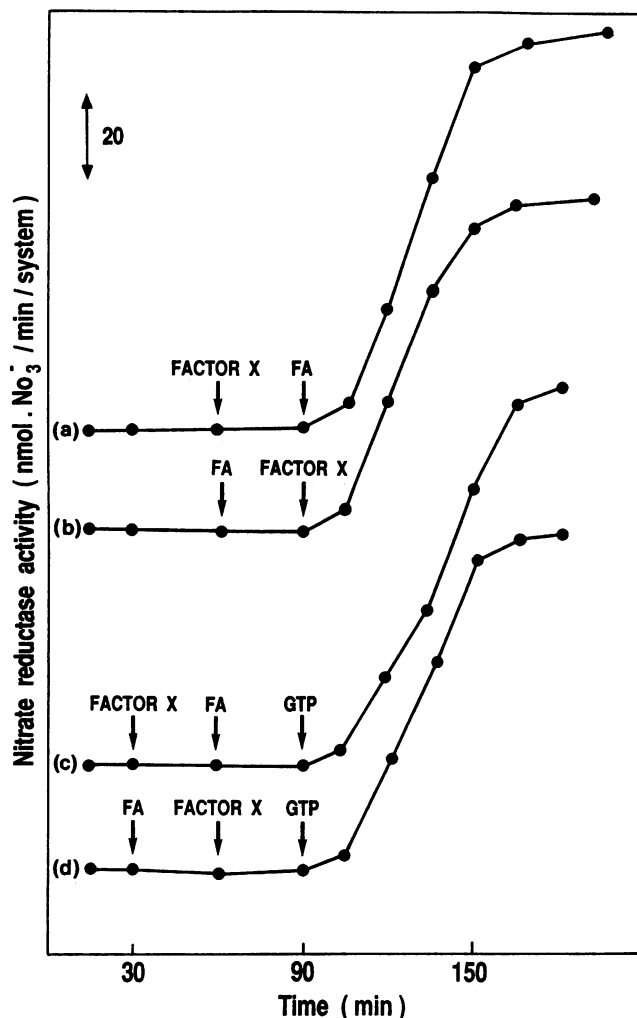


FIG. 4. The order of addition of components does not affect activation of nitrate reductase from a *chlB* mutant. (Curve a) Nitrate reductase from a *chlB* mutant (10  $\mu\text{g}$  of protein) and heated soluble fraction from a *chlB* mutant (300  $\mu\text{l}$ ) were placed in the incubation mixture. Protein FA (30  $\mu\text{g}$  of protein) and factor X (80  $\mu\text{g}$  of protein) were added at the indicated times. The final volume after the addition of protein FA and factor X was 500  $\mu\text{l}$ . (Curve b) Same conditions as for curve a, but protein FA and factor X were added in the reverse order. (Curve c) Nitrate reductase from a *chlB* mutant (10  $\mu\text{g}$  of protein) was placed in the incubation mixture. Protein FA (30  $\mu\text{g}$  of protein), factor X (80  $\mu\text{g}$  of protein),  $\text{MgCl}_2$  (1  $\mu\text{M}$ ), and GTP (to give final concentration of 1 mM) were added as indicated. The final volume was 500  $\mu\text{l}$ . (Curve d) Same conditions as for curve c, but protein FA and factor X were added in the reverse order.

Heated crude extract from a *chlB* mutant can substitute for GTP in the nitrate reductase activation process. The rate of activation was the same, regardless of whether heated crude extract from a *chlB* mutant or 1 mM GTP was present (Fig. 4). In both cases, a short initial lag phase was present. The lag phase remained unaltered, whether the activation was initiated by factor X, protein FA, or GTP. This behavior suggests that more than one step is involved in activation and that the availability of the active substance in the heated crude extract does not limit the rate of activation.

The effect of variation of factor X concentration on the activation is shown in Fig. 5. The rate of activation is limited

TABLE 3. Nucleotide specificity of activation of nitrate reductase from a *chlB* mutant

Nucleotide addition <sup>a</sup>	Activation <sup>b</sup>
GTP	
1 mM	85
2 mM	89
5 mM	11
GDP, 1 mM	72
GMP, 1 mM	19
ATP	
1 mM	$\leq 1$
2 mM	4
5 mM	3
ADP, 2 mM	$\leq 1$
AMP, 2 mM	$\leq 1$

<sup>a</sup> The nucleotides were added to the standard activation mixture containing purified nitrate reductase from a *chlB* mutant (10  $\mu\text{g}$ ), protein FA (30  $\mu\text{g}$ ),  $\text{MgCl}_2$  (1  $\mu\text{M}$ ), and factor X (80  $\mu\text{g}$ ). Incubation and assays were performed as described in Materials and Methods.

<sup>b</sup> Nitrate reductase activity expressed as nanomoles of nitrate reduced per min per reaction mixture.

by the amount of factor X present under the conditions used. The extent of activation, however, was independent of factor X. This behavior is consistent with factor X acting as a catalyst in the process.

**Chlorate resistance loci are not required for factor X activity.** Mutations in the chlorate resistance loci lead to the inability to synthesize active molybdoenzymes in *E. coli*. Factor X activity is clearly required for the activation of nitrate reductase from a *chlB* mutant, so it would be reasonable to suppose that some chlorate-resistant mutants would be defective in this activity. We explored this possibility by

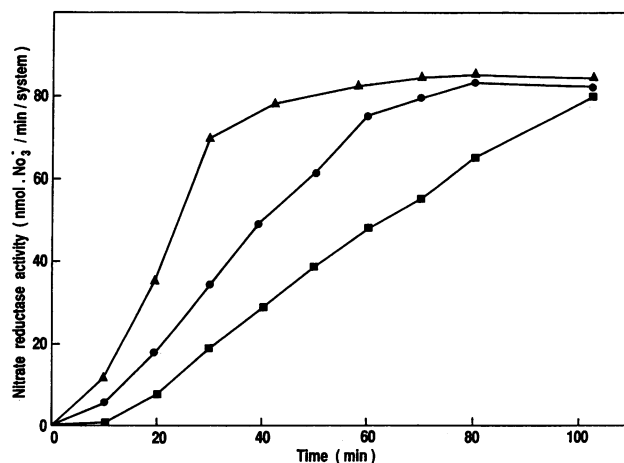


FIG. 5. Effect of factor X concentration on its ability to activate nitrate reductase from a *chlB* mutant. The mixture (total volume of 500  $\mu\text{l}$ ) contained 10  $\mu\text{g}$  of nitrate reductase from a *chlB* mutant, 30  $\mu\text{g}$  of protein FA,  $\text{MgCl}_2$  (1  $\mu\text{M}$ ), 1 mM of GTP, and different amounts of factor X (40  $\mu\text{g}$  [■], 80  $\mu\text{g}$  [●], and 160  $\mu\text{g}$  [▲]). The mixtures were incubated for the time indicated, and nitrate reductase activity was assayed as described in Materials and Methods. Nitrate reductase activity is expressed in nanomoles of nitrate reduced per min per reaction mixture.

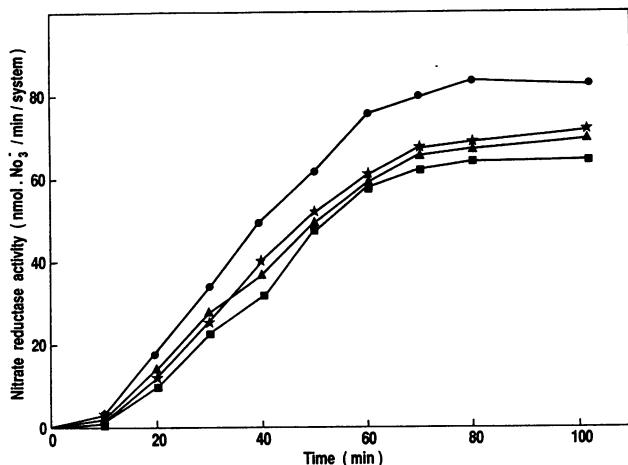


FIG. 6. Factor X activity from strains with defective chlorate resistance loci. The activation mixtures contained nitrate reductase from a *chlB* mutant (10  $\mu$ g of protein), protein FA (30  $\mu$ g of protein),  $MgCl_2$  (1  $\mu$ M), and GTP (1 mM) with factor X (80  $\mu$ g of protein) from the mutant strain indicated. The final volume for the reactions mixture was 500  $\mu$ l. Incubation and nitrate reductase assay were carried out as described in Materials and Methods. Activities are expressed as nanomoles of nitrate reduced per min per reaction mixture. Symbols: ★, mutant LCB 162 A (*chlA narG*); ●, mutant LCB 162 B (*chlB narG*); ▲, mutant LCB 162 E (*chlE narG*); ■, mutant LCB 162 G (*chlG narG*).

assessing factor X activity in a series of strains, with each strain carrying an insertion mutation at a different chlorate resistance locus. All the strains used also possessed an insertion in *narG*, the structural operon of nitrate reductase, which may be present in factor X preparations from the other chlorate-resistant strains. It is clear from Fig. 2a that factor X activity does not require a fully functional *chlB* locus.

Factor X activity is present in a *chlA* strain (Fig. 2b). In strains containing protein FA, the soluble extract must be subjected to gel filtration, which separates factor X from protein FA, in order to specifically detect of factor X activity. Figure 2b shows that factor X activity is clearly separated from protein FA following gel filtration on Sephadex G75. Similar manipulations were performed on strains with insertion mutations at *chlE* and *chlG*. Both strains exhibited factor X activity. Figure 6 shows that equivalent levels of activation of *chlB* nitrate reductase was achieved with factor X preparations from any of the *chlA*, *chlB*, *chlE*, and *chlG* strains. These experiments indicate that factor X is unlikely to be a product of any known chlorate resistance locus. Furthermore, since molybdopterin is absent from *chlA* and *chlE* strains, factor X cannot function in the activation process by supplying molybdopterin or molybdenum cofactor to the activation system. This is supported by our failure to detect these substances in factor X preparations by fluorimetric analysis (Fig. 1).

## DISCUSSION

A full understanding of the mechanism of activation of nitrate reductase from a *chlB* mutant requires the ability to effect the activation *in vitro* with defined isolated components. This report describes some progress toward achieving this objective. The inactive nitrate reductase present in a *chlB* mutant after purification to apparent homogeneity has

been activated. We have shown that the activation requires, in addition to protein FA, Mg-GTP, and a previously unsuspected protein termed factor X. Protein FA is a substantially purified preparation thought to be the active product of the *chlB* locus (21, 26). Factor X activity has been partially purified from the soluble cellular fraction.

We previously showed that the activation of nitrate reductase from a *chlB* mutant required a heat-stable low-molecular-weight substance other than the molybdenum cofactor (5). We showed that the concentration of this substance was increased by the addition of Mg-ATP to the crude activation mixture, but the compound was itself distinct from ATP. The present demonstration that Mg-GTP fulfills this role is in accord with our earlier work. The ubiquitous nucleotide transferases would increase GTP levels in crude extracts upon ATP addition. Interestingly, 5 mM GTP is inhibitory to the activation, which explains why we failed to identify GTP in our earlier work, since in that study (5), GTP was used only at a concentration of 5 mM. Our present work cannot exclude the possibility that GDP is able to act directly as GMP donor, since GDP also promoted effective activation. The GDP result could be explained by the likely presence of guanylate kinase in the factor X preparation.

Johnson et al. (18) demonstrated that *chlB* mutants lack molybdopterin guanine dinucleotide but possess molybdopterin. This result strongly suggests that *chlB* mutants are defective in the ability to add the GMP moiety to molybdopterin. Our demonstration of the involvement of GTP in the activation process is in accord with the work of Johnson et al. (18) and indicates that GTP is the likely donor of GMP in molybdopterin guanine dinucleotide synthesis.

The most direct explanation of these findings is that molybdopterin guanine dinucleotide is synthesized during the activation of nitrate reductase from a *chlB* mutant. Protein FA activity is absent in all *chlB* mutants examined and present in all other chlorate resistance mutants tested (21). Protein FA clearly requires a functional *chlB* locus for its activity, and this activity is obviously required for molybdopterin guanine dinucleotide formation. In this study, we have shown that nitrate reductase from a *chlB* mutant is the only component in the activation mixture that contains pterin-like material. A straightforward explanation for the activation mechanism is that molybdopterin guanine dinucleotide is synthesized on nitrate reductase from bound molybdopterin and Mg-GTP. This simple interpretation, however, is complicated by the requirement for two distinct activities, those of protein FA and factor X. Furthermore, our initial kinetic analysis indicates that more than one step is involved in the activation process. Protein FA and factor X are clearly involved in the late posttranslational maturation steps in the biosynthesis of nitrate reductase. The resolution of the respective roles of protein FA and factor X in the process requires further experimentation.

It is surprising that factor X activity appears unimpaired in mutants with defects all of the known chlorate resistance loci. However, all of the chlorate resistance loci encode multiple gene products, and we cannot formally exclude the possibility that despite the use of insertion mutations, some genes at the mutated loci may remain functional. The presence of factor X activity in the *chlA* mutant indicates that it is distinct from protein FA, which we have previously implicated in the activation of nitrate reductase from a *chlA* mutant (10).

All known molybdoenzymes in *E. coli* are defective in *chlB* mutants, and we have shown that their *in vitro* activation requires protein FA activity (7). The activation process

studied in this communication is therefore almost certainly common to the biosynthesis of all *E. coli* molybdoenzymes.

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